Snm as a Photolabile Protecting Group for the Cysteinyl Radical − Direct Evidence from Time-Resolved IR and UV/Vis Spectroscopies

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Keywords: IR spectroscopy / Cysteine / Disulfide bridge / Radicals / Kinetics / Photochemistry

The cysteinyl radical and the methyl(phenyl)thiocarbamic acid radical have been generated by laser flash photolysis (266 nm) of BOC–Cys(Snm)–OH in acetonitrile and characterized both by time-resolved (IR and UV/Vis) and standard spectroscopic methods (UV, IR, and NMR). The mode of action of the Snm unit [methyl(phenyl)thiocarbamic acid] is similar to the so-called "caging" technique. Cleavage of the disulfide bridge by irradiation is possible in acceptable quantum yields. Time-resolved IR (TR-IR) spectroscopic experiments using the step-scan FTIR technique allowed the detection of the methyl(phenyl)thiocarbamic acid radical ($\nu_{C=O}$ = 1607.7 and 1579.3 cm^{-1}), which has a lifetime of about 3 μs. Because of the weak absorptions in the IR spectrum and the tendency for fast recombination to BOC-protected cystine, the cysteinyl radical cannot be monitored by TR-IR spectroscopy. Time-resolved UV/Vis spectroscopy revealed the formation of both of these radicals and permitted the determination of the decay rates of the methyl(phenyl)thiocarbamic acid radical in the presence of several quenchers. NMR spectroscopic studies of the photolysis of BOC–Cys(Snm)–OH allowed us to identify *N*-methylaniline, carbonyl sulfide, and BOC-protected cystine as the major products. This study clearly demonstrates that the thiol protection group Snm is a suitable photolabile protecting group. (© Wiley-VCH Verlag GmbH & Co. KGaA, 69451 Weinheim, Germany, 2003)

Introduction

Disulfide bridges play an important role in defining the three-dimensional structure of peptides and proteins. During the last few years, extensive investigations on the photoreaction of disulfide bridges in relatively small biomolecules have been undertaken.[1] The disulfide cleavage can be performed by various techniques. Direct irradiation yields the resulting cysteinyl radicals only in poor quantum yields, although it is known[2−5] that aryl disulfide bonds can be cleaved in less than 100 ps by ultrashort UV laser pulses (λ_{exc} = 270 nm). A major problem is the fate of the cysteinyl radical, and efforts have been made to stabilize this radical so as to avoid rapid recombination.[6,7] Several techniques have been reported to monitor the radicals.[8−12]

Here we report on the photochemical generation and indirect spectroscopic detection of cysteinyl radicals. Thiyl radicals, such as the cysteinyl radical, show only very weak absorptions in the IR spectrum, and thus the direct detection of the cysteinyl radical by the comparatively insensitive step-scan technique is not possible. In this paper, we present a convenient method for the generation and detection of the cysteinyl radical. We used a "caging" technique, which has been used in context with the step-scan technique before,[14] for the indirect detection of the cysteinyl radical. This technique allows the detection of radicals through a "reporter" position with a strong IR absorption next to the disulfide bridge. A variety of molecules have been investigated, but the thiol protection group Snm [methyl(phenyl)thiocarbamic acid] published by Barany et al.,[13] which can be cleaved under mild thiolytic conditions, was found to be most suitable for this application.

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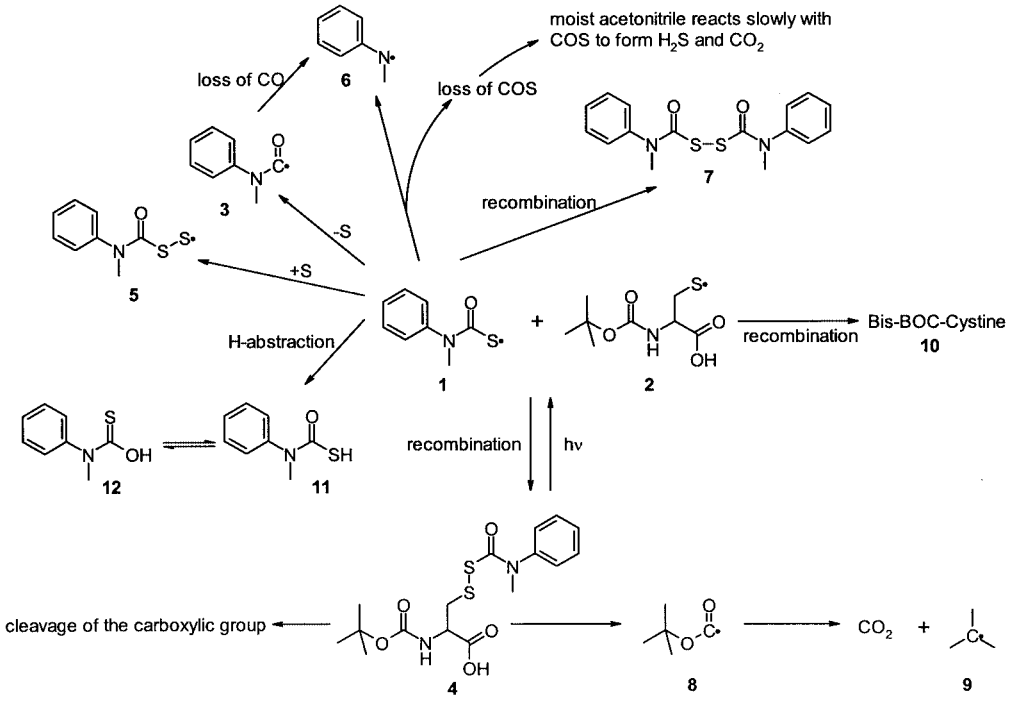
Results and Discussion

Photochemistry of BOC−Cys(Snm)−OH

Laser flash photolysis (LFP) (λ_{exc} = 266 nm) with step-scan detection of **4** in acetonitrile (1.2 mmol, Ar purge) resulted in the bleaching of the 1678.2 cm^{-1} absorption of **4** and the formation of a transient species with an absorption at 1607.7 cm^{-1}. The lifetime of the transient species was estimated to be 2.5±0.5 μs. Both signals were detected during the rise time (25 ns) of the step-scan setup. A complete measurement covers a time range of 20 μs, within which no further signals were detected. A partial recovery of the bleached signal after 20 μs indicates the formation of a further product. Infrared bands in this region are not characteristic of acyl radicals.[15] A possible fragmentation pattern of **4** is shown in Scheme 1.

Several products and fragmentation pathways can be disregarded immediately. A cleavage of the carboxylic acid group is energetically unfavourable. The loss of the BOC protection group would result in the formation of the *tert*-butoxycarbonyl radical **8**, which undergoes rapid decarb-



Scheme 1

oxylation yielding the *tert*-butyl radical **9**. The detection and kinetics of these radicals has been reported by our group recently.[16] Since no evidence for them is found in our spectra, both fragmentation pathways can be disregarded. A suitable site for bond breakage is the disulfide bridge. The cleavage of the S−S bond would lead to the methyl(phenyl)thiocarbamic acid radical **1** and the cysteinyl radical **2**. Because of the lack of an intense IR chromophore and the tendency for rapid recombination, radical **2** cannot be monitored by the step-scan technique, but the methyl(phenyl)thiocarbamic acid radical **1** seems a promising candidate for it (Figure 1).

In order to confirm the assignment, the vibrational spectrum of **1** was calculated using density functional theory and ab initio methods [UB3LYP/6-311++G(d,p) and UMP2/6-31+G(d,p)] (Figure 2). The calculated infrared spectrum predicts additional bands should exist in the spectral region of interest. To verify the calculation, the transparency of the cell was increased by reducing the spacer size (0.05 mm). In addition to the bands at 1607.7 cm^{-1} and 1678.2 cm^{-1}, a new absorption is observed at 1579.3 cm^{-1}, which decays with the same kinetics as the band at 1607.7 cm^{-1}. The results obtained are in good agreement with the

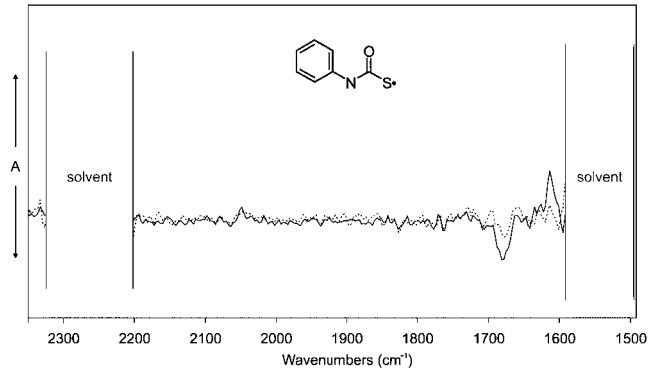


Figure 1. Time-resolved difference IR spectra (time resolution 500 ns, spectral resolution 6 cm^{-1}, spacer size 0.2 mm) showing the photochemistry of **4** in argon-purged acetonitrile; bands appearing on irradiation (λ = 266 nm) are pointing upwards, bands disappearing are pointing downwards; black: spectrum 500 ns after irradiation; dotted: spectrum after 20 μs

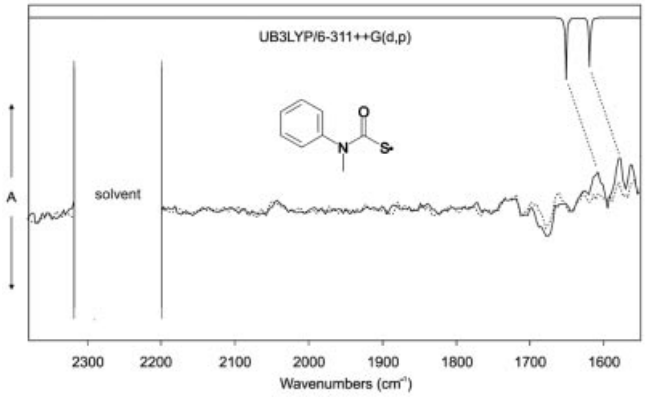


Figure 2. Top: calculated vibrational spectrum of **1** [UB3LYP/6-311++g(d,p), unscaled]; bottom: time-resolved difference IR spectra (time resolution 500 ns, spectral resolution 6 cm^{-1}, spacer size 0.05 mm) showing the photochemistry of **4** in argon-purged acetonitrile; bands appearing on irradiation (λ = 266 nm) are pointing upwards, bands disappearing are pointing downwards; black: spectrum 500 ns after irradiation; dotted: spectrum after 20 μs

theoretical predictions [the C=O stretching vibrations of **1** are calculated at the UB3LYP/6-311++G(d,p) level of theory to be 1619.4 and 1651.3 cm^{-1}].

The IR spectra of the radicals **3** and **5** − as well as the trapping products **7**, **11**, and **12**, as potential secondary products of **1** − also were calculated using DFT and ab initio methods [(UB3LYP/6-311++G(d,p) and UMP2/6-31+g(d,p)]. None of the calculated spectra matched the experimental results. Radical **3** is expected to decarbonylate rapidly to the aminyl radical **6**, but neither radical **3** nor carbon monoxide, with a characteristic and strong IR absorption at 2140 cm^{-1}, was found in our spectra. Because of the lack of strong IR chromophores, the aminyl radical **6** is not expected to be observable by step-scan FTIR. This radical will be discussed in context with the time-resolved UV/Vis experiments.

It is known[17,18] that the acetylthiyl radical and the benzoylthiyl radical undergo β-fragmentations:

$CH_3COS^{\cdot} \rightarrow CH_3^{\cdot} + COS$

$PhCOS^{\cdot} \rightarrow Ph^{\cdot} + COS$

The $PhCOS^{\cdot}$ radical undergoes β-fragmentation to form the phenyl radical and carbonyl sulfide with a rate constant of $8.5 \cdot 10^3$ s^{-1} at room temperature;[17] for $CH_3COS^{\cdot}$ this rate constant is $6.6 \cdot 10^4$ s^{-1}.[18] Thus, we expect a similar rate constant for the loss of COS from radical **1**. Within the time range of 20 μs, however, no COS signal (2049.5 cm^{-1})[19] was observed in the IR spectra. This observation might be explained by the slow hydrolysis of COS to CO_2 and H_2S (at 25 °C, the pseudo-first-order rate constant is 0.0011 s^{-1}).[20]

Because the intial step − the loss of COS − takes place on a time scale too slow to be monitored by our step-scan measurement, the subsequent step − very slow hydrolysis to carbon dioxide − also can not be observed directly. The activation barrier for the loss of COS from **1** was calculated using DFT and ab initio methods to be 12.5 kcal/mol [(UB-3LYP/6-311++G(d,p)] (Figure 3).

Thus, the loss of COS should take place at room temperature, but on a much longer time scale than the observed decay of **1**. The stability of **1** was confirmed by further independent techniques (LFP, liquid nitrogen Cryostat and product studies after preparative irradiation). The formation of radical **1** reveals clearly that the Snm protection group is photolabile. The cysteinyl radical **2** must be formed as the second fragment of the cleavage of **4**. Radical **2** could either undergo hydrogen abstraction from the solvent or recombine with **1** to give the starting compound **4**. A further possibility is the dimerization to yield cystine **10**. We performed a TR-IR spectroscopic experiment under 1 bar of oxygen in order to examine the quenching/oxidation chemistry of **1**. The spectra obtained in this experiment showed a variety of signals with a much more complex photochemistry and an inferior S/N ratio than in the argon-purged experiments.

To confirm that a loss of COS from **1** takes place on a relatively long time scale, and that the cysteinyl radical **2** is formed, we used time-resolved UV/Vis spectroscopy. A loss of COS from radical **1** forms the aminyl radical **6**. Aminyl

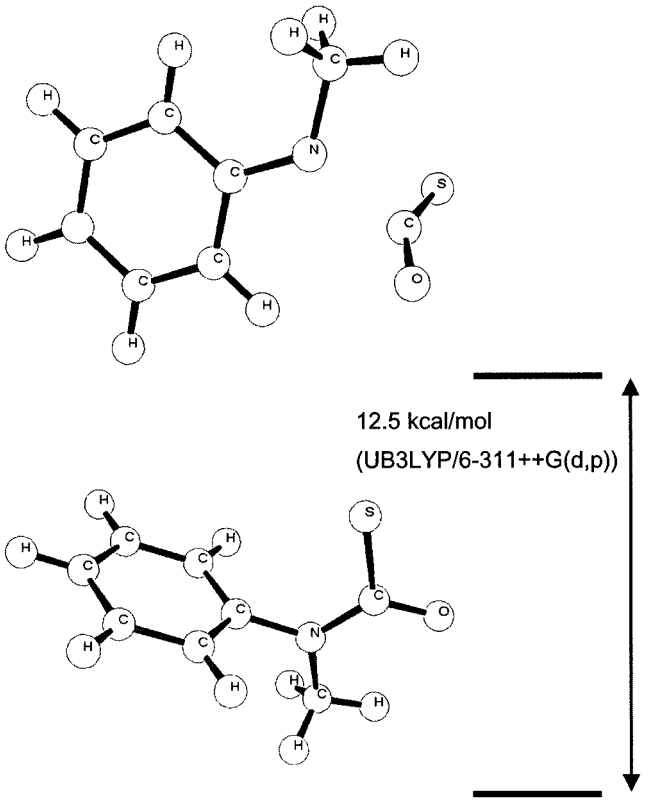


Figure 3. Structures of **1** and the transition state for the loss of calculated at the UB3LYP/6-311++G(d,p) level of theory

radicals have been examined extensively by several research groups using LFP.[21,22] These radicals generate transient absorptions in the region around 400 nm. We conducted LFP experiments with **4** under the same conditions as described for the TR-IR experiments. The TR-UV/Vis spectra showed a short-lived transient species with absorptions at λ_{max} = 630 and 345 nm. Furthermore, a long-lived transient species is observed that overlaps with the absorption at λ = 345 nm (Figure 4). The transient species observed at λ = 370 and 600 nm (Figure 5) decayed with second-order kinetics, yielding a half-life of approximately $t_{1/2} = 5.3 \cdot 10^{-6}$ s.

At 295 nm the long-lived transient decayed with $k = 1.8 \cdot 10^4$ s^{-1} (τ = 55 μs) in a first-order reaction. In agreement with the lifetime obtained from the TR-IR experiments, we assigned the short-lived transient species to the methyl(phenyl)thiocarbamic acid radical **1** and the long-lived species to the cysteinyl radical **2**. In order to confirm our assignment, the UV/Vis spectra of radical **1** was calculated using density functional theory [(UB3LYP/6-311++G(d,p)] on the previously optimized structure of the radical. The calculated absorptions are in good agreement with the experimental results (Table 1).

Our time-resolved UV/Vis measurements cover the time ranges up to 75 μs. Within this time range, we did not observe the appearance of an absorption in the region of $\lambda \approx$ 400 nm indicating the formation of aminyl radical **6** by the loss of COS, a situation that is in accordance with the con-

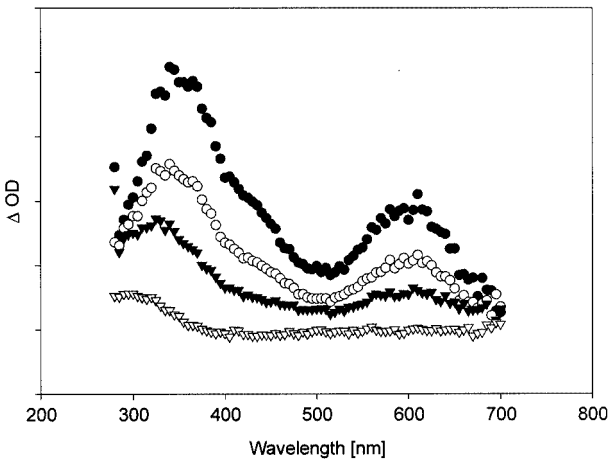


Figure 4. Time-resolved UV/Vis spectra [traces recorded after 1 μs (black dots), 5 μs (white dots), 17 μs (black triangles), and 75 μs (white triangles), spectral resolution 5 nm, cell size 1.0 cm] displaying the photochemistry of **4** in argon-purged acetonitrile

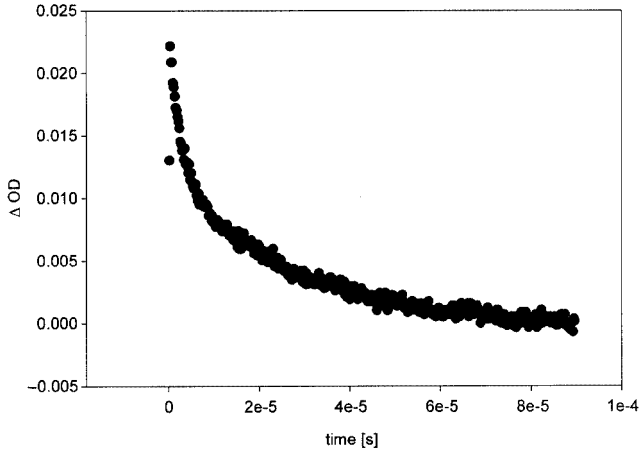


Figure 5. Transient decay trace, recorded at λ = 370 nm, upon laser flash photolysis (λ_{exc} = 266 nm) of **4** in acetonitrile at ambient temperature

Table 1. Experimental and calculated UV absorptions of the methyl(phenyl)thiocarbamic acid radical **1**

Experimental λ [nm]	Calculated λ [nm][a]
345.0	325.9
465.0	478.8
630.0	621.6

[a] UB3LYP/6-311++g(d,p).

clusions from the TR-IR measurements. According to published LFP data on cystine and its derivatives, the cysteinyl radical should exhibit an absorption maximum at 330 nm.[8,9] We assume that this absorption overlaps with the absorption of **1** at λ = 345 nm, which has to be taken into account when evaluating the kinetic data. It has been reported in the literature that the cysteinyl radical can abstract sulfur atoms yielding the CysSS· radical with a transient absorption at λ ≈ 380 nm.[8,9] The formation of the CysSS· radical, however, is energetically not favoured.

Thiyl radicals add quickly to double bonds in an anti-Markovnikov fashion.[23−25] We performed quenching experiments and measured the rate constant for the reaction of **1** with 1,4-cyclohexadiene (1,4-CHD) by determining the decay rate of **1** in the presence of 1,4-CHD at seven different concentrations (Figure 6). The rate constant for this reaction is $3.5 \cdot 10^6$ L·mol^{-1}·s^{-1}. Similarly, the rate constant for the reaction of **1** with methyl methacrylate (MMA) was determined to be $k = 1.95 \cdot 10^7$ L·mol^{-1}·s^{-1} by determining the decay rate of **1** in the presence of five different concentrations of MMA (Figure 7).

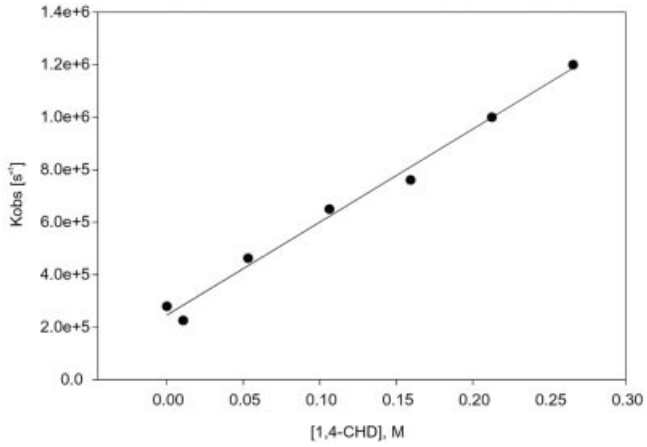


Figure 6. Decay rate of **1** in the presence of seven different concentrations of 1,4-cyclohexadiene

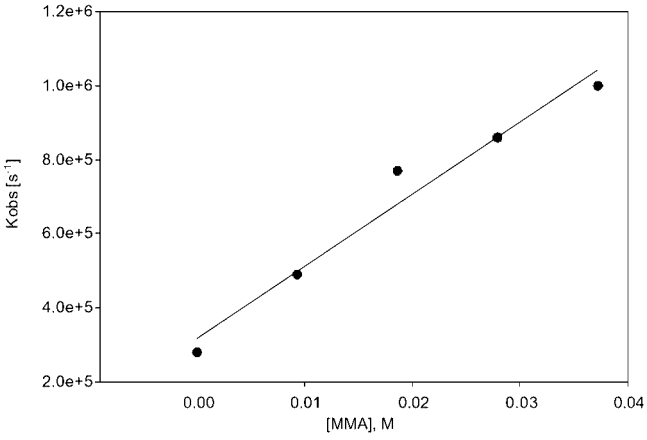


Figure 7. Decay rate of **1** in the presence of five different concentrations of MMA

Monochromatic UV irradiation (λ_{exc} = 266 nm) of **4** in degassed acetonitrile at 230 K resulted in slow bleaching of a shoulder at λ = 284 nm. Simultaneously, a band at λ = 330 nm with the same kinetic behaviour is formed. This new absorption at 330 nm is in good agreement with the expected absorption for the cysteinyl radical **2**.

We performed a product study to confirm the formation of the cysteinyl radical **2**. A degassed acetonitrile solution

of **4** in a sealed quartz cell was irradiated for 1 h with the irradiation of an Nd:YAG laser at 266 nm. After irradiation, the previously colourless solution had turned slightly brown and the odour of thiol-containing compounds was sensed. A ^{1}H NMR spectrum in [D$_6$]DMSO revealed that clean photochemistry had occurred. Under these conditions, the loss of COS takes place as indicated by the formation of *N*-methylaniline. To trap the COS we repeated the experiment in a fused quartz NMR tube. We recorded ^{1}H NMR, ^{13}C NMR, and HMQC spectra. All signals in the ^{1}H NMR (Table 2) and ^{13}C NMR (Figure 8) spectra can be assigned clearly to COS, *N*-methylaniline, BOC-protected cystine **10**, and traces of nonphotolized **4**. There is no evidence that **7** is formed since the characteristic shift of the −NMe group at δ = 3.36 ppm[26] is missing.

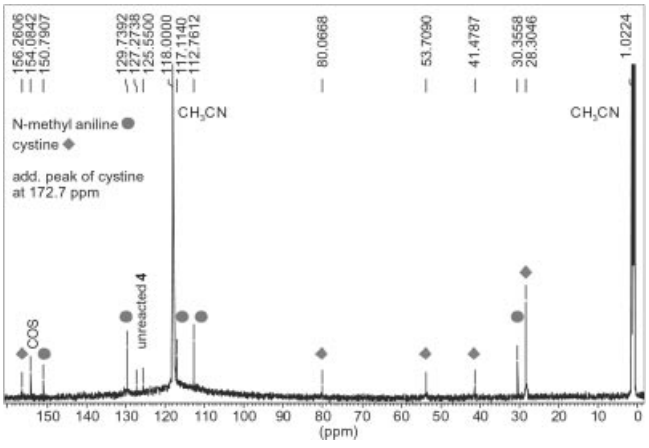


Figure 8. ^{13}C NMR spectra (150 MHz) after preparative photolysis (1 h, 266 nm, 10 Hz, 0.6 W s^{-1}) of **4** in degassed CD$_3$CN in a fused quartz NMR tube

The lability of the Snm protection group towards irradiation can be monitored by a simple experiment. Exposure of a normal NMR sample of **4** (for example in [D$_6$]DMSO) to daylight for several weeks resulted in the same colour change to slightly brown as was observed during the laser irradiation. To confirm the ability of Snm to act as a photolabile protecting group, we examined the irradiated sample by FTIR spectroscopy. After irradiation, the IR spectra showed three new bands in the carbonyl region: a strong absorption at 1753.7 cm^{-1} and two very strong bands at 1715.6 and 1512.7 cm^{-1}. These signals are assigned to the three prominent vibrations of the BOC-protected cystine **10** (Table 3), and is in good agreement with literature data.[27]

Table 3. IR absorptions of photoproduct **10** in solution (CH$_3$CN)

Exptl. $\tilde{\nu}$ [cm^{-1}]	Exptl. $\tilde{\nu}$ [cm^{-1}][27]	Assignment
1753.7	1743	$\nu_{C=O}$ CO_2H
1715.6	1718	$\nu_{C=O}$
1512.7	1509	Amide II

In conclusion we have shown that Snm can be used as a photolabile protecting group. Photolysis leads, in good yields, to the interesting methyl(phenyl)thiocarbamic acid radical **1** and the well-known cysteinyl radical **2**, which undergo various subsequent reactions (e.g., **2** dimerizes to form cystine **10**). Thus, the precursor molecule **4** can be considered as a "caged" cysteinyl radical **2**. In view of the many biochemical functions of cysteinyl radicals, this protecting group and these spectroscopic techniques might find applications in the exploration of biochemical reaction pathways.

Experimental Section

Step-Scan FTIR: For time-resolved measurements we have used a set-up that has been described previously.[16] Excitation of the sample is carried out by the 4th harmonic (266 nm) of an Nd-YAG laser (Spectra-Physics Quanta-Ray LAB 100) with a repetition rate of 10 Hz. To avoid thermal effects and shockwaves, the laser energy is attenuated to between 15 and 28 mJ/pulse by an external attenuator. All measurements were carried out with 6 cm^{-1} resolution in

Table 2. ^{1}H NMR spectroscopic data (600 MHz) of the products obtained after preparative photolysis (1 h, 266 nm, 10 Hz, 0.6 W·s^{-1}) of **4** in degassed CD$_3$CN

Exptl. δ [ppm]	Assignment	Exptl. δ [ppm][a]	Exptl. δ [ppm][27]
1.42	BOC CH_3, of **10** and unchanged **4**		1.47
2.74	CH_3, methylaniline	2.82	
2.97	Cys β-CH_2, of **10** and unchanged **4**		
3.27	Cys β-CH_2, of **10** and unchanged **4**		3.25
3.33	NCH_3 of unchanged **4**		
4.39	NH, methylaniline	4.30	
4.46	Cys α-CH, of **10** and unchanged **4**		4.47
5.73	NH of **10**		5.78
	COOH of **10**		6.28
6.15	NH of unchanged **4**		
6.58	Aromatic ring, methylaniline	6.69	
7.12	Aromatic ring, methylaniline	7.26	
7.37	Aromatic ring of unchanged **4**		
7.47	Aromatic ring of unchanged **4**		

[a] Recorded as a reference (200 MHz, CD$_3$CN).

a spectral range between 0 and 3160 cm^{-1}, resulting in an interferogram containing 1060 points. The signal is averaged 20 times per sampling position. In all experiments, a time range of 20 μs is recorded with a resolution of 25 ns. After FT, the average of 20 successive spectra is calculated to achieve a better signal-to-noise ratio, resulting in an effective time resolution of 500 ns. If necessary, this figure can be improved by averaging a smaller number of spectra. The sample is dissolved (between 1.0 and 6.0 $g \cdot L^{-1}$ depending on the layer thickness of the cell) in acetonitrile (Riedel-de Haën, spectroscopic grade) and purged with argon for at least 45 min.

Laser Flash Photolysis: A standard LFP set-up was used, consisting of a Spectra-Physics Quanta-Ray LAB 130 Nd-YAG laser, operated at 1 Hz and 266 nm (50 mJ/pulse, 8 ns pulse duration), a pulsed Xe arc lamp by Müller, Germany, a TRIAX 180 monochromator coupled to a photoelectron multiplier tube, and a LeCroy 9361 digital oscilloscope. The entire set-up was controlled from a personal computer using LabView software. Data evaluation was performed using SigmaPlot software. In order to avoid depletion of the precursor and product build-up, a flow-cell was used. Quenching experiments were performed in the same way, except that the flow-cell was replaced by a static cell. The concentration of **4** was adjusted so that an optical density of 0.3 was achieved at the laser wavelength used for excitation.

Calculations: Calculations were performed with the Gaussian98[28] suite of programs. Transition states, energies, vibrational and UV spectra were calculated using B3LYP or MP2 theory with a 6-311++G(d,p) or 6-31+G(d,p) basis set.

Product Studies: Photolyses were performed using acetonitrile or [D_3]acetonitrile and an Nd-YAG laser (Spectra-Physics Quanta-Ray LAB 100) operating at λ_{exc} = 266 nm with a repetition rate of 10 Hz. The preparative photolysis was carried out with 0.6 $W \cdot s^{-1}$ for 1 h. Samples (0.04 mol/L) were poured in quartz NMR tubes and degassed by the use of a supersonic bath and the "freeze-and-pump" technique. The tubes were fused and ^1H, ^{13}C, and HMQC NMR spectra were taken before and after the photolysis (Bruker, Avance DPX 200, 200.13 MHz and Bruker, Avance DRX 600, 600.13 MHz).

Nitrogen Cryostat: Measurements were carried out in acetonitrile solutions at 230 K in a liquid-nitrogen cryostat (Oxford, DN1714 equipped with Suprasil windows). Samples were poured into self-made quartz cuvettes (length 60 cm, diameter 1.0 cm), degassed in the same way as described for the preparative photolysis and sealed with a stopper. For irradiation, we used a high-pressure mercury lamp (Mercury Light Source 200, Bausch & Lomb, Rochester, NY, USA) with a monochromator (Bausch & Lomb, grating 1200 grooves/mm) operating at λ_{exc} = 265 nm. UV spectra were recorded every minute for 1 h with a diode-array spectrometer (Hewlett−Packard, HP 8452) in the spectral range 210−700 nm with a resolution of 2 nm. To avoid influences on the sample by the deuterium lamp of the spectrometer, the scan time was set to 0.5 s.

Synthesis: The precursor BOC−Cys(Snm)−OH was synthesized according to a literature procedure described by Barany et al.,[13] except that the workup was modified slightly.

Acknowledgments

C. K. is indebted to PD Dr. G. Bucher for his introduction and advice to the LFP technique. This work was financially supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 452) and the Fonds der Chemischen Industrie.

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Received August 14, 2002
[O02468]